STUDIES ON THE AETIOLOGY OF NON-DIPHTHERITIC CROUP

With Special Reference to the Study of a Virus Isolated from Children with this Disease

By

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The present paper comprises a summary of earlier investigations reported in the following papers:

I. Aetiology of non-diphtheritic croup I. Bacteriologic and serologic investigation. *Acta Pædiatrica. 47*, 265, 1958.

II. Non-diphtheritic acute subglottic laryngitis (Croup) in children. A family study. *Int. Arch. All. and Appl. Immunol.* 1958. In press.

III. Together with T. WESSLÉN. Recovery of a cytopathogenic agent from patients with non-diphtheritic croup and from day-nursery children. I. Properties of the Agent. *Arch. für Virusforsch. 8*, 77, 1958.

IV. Idem II. Physico-chemical and growth characteristics. *Arch. für Virusforsch. 8*, 192, 1958.

V. Idem III. Studies on the haemagglutination and haemagglutination-inhibition of the agent. *Arch. für Virusforsch. 8*, 1958. In press.

VI. Association between a recently isolated virus and an upper respiratory disease epidemic in a day-nursery. *Arch. für Virusforsch. 8*, 204, 1958.

VII. Experiments in human adults with a recently isolated virus associated with respiratory disease. *Arch. für Virusforsch. 8*, 1958. In press.

VIII. Aetiology of non-diphtheritic croup. II. Virologic investigation. *Acta Pædiatrica. 47*, 1958. In press.

In the following these papers will be referred to using the Roman numerals given above.

Introduction

The term "croup" has been generally applied to any affection of the larynx and trachea in children producing difficult and noisy respiration and a hoarse cough. Before the great pandemic of influenza in 1918 these affections were regarded as due to diphtheria, but since the recognition of a non-diphtheritic type numerous investigations have reported different bacterial organisms responsible for this syndrome, as reviewed by Neffson (17). Differentiation of different clinical syndromes according to aetiologic conditions has been postulated (20), but the clinical and therapeutic advantages of such differentiation have been questioned (9, 20). Haemolytic streptococci, pneumococci, *Staphylococcus aureus pyogenes*, and *Hemophilus influenzae type B* are among the bacterial species reported as possible causative agents (16, 14, 15, 13, 25). Rabe, however, showed in controlled experiments that of the bacterial species only *H. infl. type B* could be regarded as the causative organism in 8 per cent of his cases, and in these instances the syndrome was of a supraglottic type (22). The most common type of non-diphtheritic croup, the subglottic, was referred to as virus croup by Rabe without the necessary justification of an aetiologically associated viral agent (21). Recently, Chanock and Beale *et al.* have reported the isolation in tissue cultures of a viral agent related to influenza viruses from cases of acute laryngotracheobronchitis (2, 4). In addition to the infectious precipitating agent widely believed to exist in this syndrome, some investigators report an allergic factor in some cases (11). The genetic or familial occurrence does not seem to have been thoroughly investigated, in spite of a high recurrency frequency of this disease.

The introduction of tissue culture in the field of virus research according to the methods developed by Enders, Weller and Robbins (23), and later by Dulbecco and Vogt (6) and simplified by Youngner (28), offered great advantages in the search for viral agents possibly associated with a disease under study. The acute respiratory infections excluding influenza, were mainly studied by transmission experiments in humans before the introduction of these methods; subsequently, adenoviruses were isolated and an association between some types of febrile acute upper respiratory infections and these viruses has been established clearly (for a review see 24). Quite recently viral agents (18, 19) have been recovered from non-febrile acute respiratory infections, e.g. common cold types of infection (5) but further investigations must be awaited before a relationship between the agents and the disease can be proved to exist. Since the aetiology of the non-diphtheritic croup syndrome was reported to differ, a study was undertaken to try to evaluate the rôle played by bacteria and viruses in the croup syndrome.

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Plan of Investigation

The study was first devoted to the rôle played by bacteria in the croup syndrome. To evaluate this problem, a bacteriologic study was undertaken of 269 admittances with this disease during a 2½-year period (I). Then it was considered necessary to ascertain whether an aetiologic factor other than the infectious one was involved. A familial study was carried out by questionnaires in a group of families in which one of the children had suffered non-diphtheritic croup, and a control group of families were subsequently investigated in the same way (II).

Finally, a virologic investigation of the croup-children was performed. To establish the rôle of the viruses, recovered, two further groups of children were investigated at the same time (VIII). During this investigation a virus was found, called Uppsala-virus (U-virus), which to the best of the knowledge of the authors was distinct from other known viruses. It was therefore considered of primary importance to study the virus from the theoretical point of view. At first the general properties of the agent were studied, including the separation of the U-virus from other known viruses, with any possible relationship, and the serological relationship between the different strains of U-virus isolated (III).

Further, the physico-chemical and growth characteristics were briefly examined (IV); the characteristics of a haemagglutinin of U-virus were investigated, as was the inhibition of this haemagglutinin by serum and some other biological fluids in current use in the cultivation of viruses (V).

During the course of these investigations some results suggested an association of the U-virus with mild respiratory disease. To establish this association further, a virologic study was performed on an epidemic of upper respiratory disease occurring in a day-nursery, in which 4 of 24 children were affected with non-diphtheritic croup symptoms (VI).

The infectivity of the virus in humans was also investigated by transmission experiments in human adults, experiments justified by the wide distribution of the U-virus in the community and by the lack of connexion with diseases other than upper respiratory infections (VIII). For the sake of clarity, the results of the investigations are presented under the followings headings: (i) Theoretical investigations of the U-virus. (ii) Aetiology of non-diphtheritic croup. (iii) Association of U-virus with mild respiratory disease.

Theoretical Investigations of the U-virus

Properties of the agent (III)

In the search for a causative viral agent in the non-diphtheritic croup syndrome, a virus called U-virus was isolated in tissue cultures of human

embryonic lung. The agent was not cytopathogenic for tissue cultures of monkey kidney at isolation attempts, although cytopathogenicity developed in this tissue after adaption. The U-virus seemed to be unrelated to other known viruses such as the croup-associated virus of Chanock (CA-virus), parotitis, influenza A, B and C, Sendai, adenoviruses, poliomyelitis and Echo-viruses. Some connexion with influenza virus type A^1–FM 1 was revealed by the haemagglutination-inhibition test but not by the neutralization test. Since the preparation of the manuscript, the relationship with Echo-viruses has been further investigated, because a haemagglutinin has recently been reported for some types of these viruses (10, 12). It was found that hyperimmune sera from guinea pigs against the first 14 prototypes of Echo-viruses did not neutralize the cytopathogenic effect of 100 TCD50 (50% tissue culture cytopathogenic dose) of U-virus. Nor did U-virus hyperimmune serum from rabbits neutralize the cytopathogenic effect of 100 TCD50 of any of these types of Echoviruses.

It was also found that U-virus did not multiply in embryonated eggs or ordinary laboratory animals by different routes of inoculation; nor did U-virus multiply in ordinary bacteriologic media and media developed for the detection of pleuropneumonia-like organisms. The U-virus was capable of agglutinating human erythrocytes irrespective of group in the ABO-system, but did not agglutinate sheep, guinea-pig, chick, or fowl erythrocytes. A concentration between 4.5 to 5.5 log TCD50 of U-virus seemed to be necessary for the detection of haemagglutination.

Methods were developed to estimate antibodies against the U-virus. Neutralization tests could be performed according to general methods. Haemagglutination-inhibition tests (H-I) were performed with slight modification of the methods developed for influenza viruses. Complement-fixation tests have, however, at yet failed to detect any complement-fixing antibodies against U-virus, in spite of high concentrations of the antigen.

Nineteen strains of U-virus isolated from croup and day-nursery children were examined for serological relationship mutually. Cross-testing by neutralization and H-I test between the strains and their hyperimmune sera produced in rabbits were performed to study this problem. The results indicated that only one type of the U-virus prevailed although differences in inducing H-I antibodies against the homologous haemagglutinin were recorded between the strains.

Physico-Chemical and Growth Characteristics (IV)

To study the production of U-virus with a method giving large yields of virus, a strain of U-virus adapted to tissue cultures of monkey kidney was examined. Further, it was considered of importance to determine the stability of the agent under conditions which might be employed

for chemical purification as well as for other practical purposes. Monkey kidney cells were used throughout in this study.

The yield in tissue cultures was comparable to that of poliomyelitis and most Echo-viruses and exceeded that reported for influenza viruses in tissue cultures. The growth curves indicated that the U-virus rapidly propagated in the cultures. In titrations of infectivity, final degeneration in the lowest concentrations was obtained after 3–4 days. The resistance of the U-virus to heat and hydrogen ion concentrations was found to be intermediate between the more resistant viruses as poliomyelitis and adenoviruses and the susceptible influenza and foot and mouth disease viruses.

The rate of inactivation at 37°C and pH 7.2 was 1.0 log unit per 36 hours. The U-virus was found to be stable between pH 5 and 8 and an optimal survival occurred at pH 5.3–6.3 on prolonged incubation at 37°C. The addition of glycerine to the virus suspensions definitely increased the stability. The U-virus showed no ether susceptibility and was inactived by formalin at a slower rate than poliomyelitis and influenza-viruses.

In this study, the physical properties of the U-virus and its haemagglutinin were studied by ultracentrifugation and gradocol-membrane filtration. The results suggested the size of the virus particle to be about 75 to 115 mμ and no gross differences in size of the haemagglutinin and the virus particle emerged from these studies.

Some experiments on inactivation of the U-virus suggested that the inactivation did not follow a first order of reaction when the decrease in log infectivity titre was plotted against time in hours. This applied to the inactivation by heat at 56°C when a pool added glycerine was investigated. Further, it was found when U-virus was inactivated at 37°C at a suboptimal hydrogen ion concentration (pH 7.9). Finally, it was also observed on inactivation of the U-virus by formalin.

A statistical calculation showed, however, the curves not to deviate significantly from the course of a first order reaction.

In conclusion, these results seem to offer final proof for the viral character of the agent. The size of the agent was suggested to be about 75–115 mμ. The stability to heat and hydrogen ion concentrations was intermediate between the more resistant and the more susceptible animal viruses. Inactivation by formalin, however, followed a slower rate than for most other animal viruses.

Studies on the Haemagglutination and the Haemagglutination-Inhibition (V)

The haemagglutinin of the U-virus was studied as to optimum conditions for the haemagglutination reaction (HA) with regard to hydrogen ion concentration, influence of Ca- and Mg-ions, and salt molarity.

Inactivation at different temperatures of the haemagglutinin and the mechanism underlying HA was also investigated.

The results showed HA to be detectable in highest titres at pH 7.2 to 7.8 when the HA was performed at 4°C. At 37°C the corresponding values for the pH-optimum were 5.9 to 6.4. The reaction was found not to be dependent upon the addition of Ca- and Mg-ions to the diluents. However, high concentrations of these ions inhibited the reaction.

Results obtained on chromatography of amniotic fluids containing U-virus gave evidence of a close correlation between the haemagglutinating particle and the infectious particle inducing cytopathogenicity in tissue cultures. These findings, together with the results obtained on ultracentrifugation and gradocol-membrane filtration of U-virus (IV), definitely suggest that the HA-capacity is closely bound to the virus particle.

The mode of attack of U-virus on the erythrocytes was studied by adsorbtion-elution experiments at 4°C, 22°C and 37°C and by chromatography in columns of erythrocyte-treated samples of U-virus. The results showed that adsorbtion and elution of virus particles occurred at all temperatures tested and that the adsorbtion was favoured at 4°C. The chromatographic study revealed a higher TCD50/HA ratio in some fractions of the erythrocyte-treated sample than in the specimen which had not been in contact with the erythrocytes. These findings may indicate that a portion of the virus only posess haemagglutinating activity but no cytopathogenic capacity and that these portion remains adsorbed to the erythrocytes. The results may, however, also hypothetically suggest that the mechanism underlying HA with U-virus involves a reaction in which the virus to a certain extent looses its HA-capacity by contact with the erythrocytes. Some evidence in favour of this hypothesis was obtained when it was shown that the HA-fractions of the erythrocyte-treated sample were not eluted again from erythrocytes. Whether or not this reaction was an enzyme-substrate reaction could not be determined, evidence for and against being obtained. The haemagglutinin of U-virus seemed, however, to be distinct from that of the enzyme-active myxoviruses.

Inhibitors against U-virus haemagglutinin were found in bovine amniotic fluid and human and rabbit sera in the magnitude of 1 log inhibitory titre. These inhibitors were not affected by trypsin, cholerae filtrate or bentonite treatment according to general methods. The results of the studies on these inhibitors suggested them to be of macromolecular size and that the inhibitors of bovine amniotic fluid and sera were related.

In conclusion, the results of this investigation further strongly corroborate the hypothesis that the haemagglutinating activity of U-virus is bound to the virus particle and thus specific. Further, a hypothesis for the mode of attack of the virus on erythrocytes is advanced which, on the bases of the results obtained, suggests that the HA-capacity of

U-virus to a certain extent is lost by contact with the erythrocyte. The physico-chemical influences upon the HA-reaction are discussed and a study of inhibitors against the haemagglutinin of U-virus presented.

Aetiology of Non-Diphtheritic Group

Bacteriologic and Serologic Investigation (I)

The aim of this study was to penetrate the rôle of bacteria in the croup-syndrome. 269 admittances of this disease to the Ear, Nose and Throat Department of the University Hospital were investigated for bacteria in nose and throat on admission to the hospital, and paired sera were collected from 125 of these cases. A smaller group, comprising 55 cases were investigated for bacteria in nose and throat on discharge for comparison with the admission test. Laboratory data such as white blood cell counts, microsedimentation rate, eosinophils, and rectal body temperature on admission were also investigated. The sera were investigated for antistreptolysin, antistaphylolysin, and in 86 cases for complementfixing antibodies against *H. infl.*

In 51 per cent *Staph. aur.* were isolated on admission. Haemolytic streptococci, pneumococci, and *H. infl.* were isolated on admission in frequencies of between 3.7 and 10.3 per cent. On discharge, the following frequencies were recorded: *Staph. aur.* 62, Pneumococci, 5.6, Haemolytic streptococci 5.6, and *H. infl.* 3.7 per cent. In only a few cases were significant rises in antibody-titres obtained between the paired sera.

Further the laboratory data showed that the majority of the patients had elevated body temperature, a slight rise in microsedimentation rate, and normal counts of white cells.

No supporting evidence of any major causative rôle for bacteria in this syndrome could be adduced. This has also been shown previously by other investigators (21), although there are also several reports to the contrary (14, 15, 16). In a single case, however, a complicating *Staph. aur.* infection was verified, and in two other children probable *H. infl.* infection was observed at the time of the croup-symptoms.

Thus, the results obtained suggest the bacterial species investigated not to play any major rôle as precipitating agents in the croup syndrome of subglottic type in this series, but also that the syndrome may be complicated by bacterial infections.

Family Study (II)

This study comprised a statistical comparison of a croup and a control series according to the Weinberg geneological method (27). The croup series were chosen from cases admitted to the hospital with this disease and the control series were selected from the Children's Welfare Centres

of Uppsala. Data were collected by questionnaire to the parents of the subjects requesting information concerning the following diseases: asthma, vasomotor rhinitis, eczema, urticaria, and croup of non-diphtheritic aetiology in the subjects, the siblings and parents of the subjects, and in the siblings of the parents.

The composition of the two series rendered them comparable as regards age, place of residence, and number of relatives in two generations.

The results suggested croup not to be associated with asthma, urticaria, or vasomotor rhinitis. The incidence of croup and eczema, however, showed a significant difference in the collective relative groups in the two series. Concerning eczema, only the incidence in the collective relative groups differed significantly between the croup and the control series. For croup, however, a significant difference of some degree emerged from the comparison of the siblings and fathers of the subjects in the two series.

The differences observed do not seem to be attributable to errors attaching to the investigation, such as closer observation in the croup series or a spread of the croup-precipitating agent from subjects to siblings in the croup series. The inference is that a non-infectious factor may be involved in the majority of non-diphtheritic croup cases. An evaluation of this factor must await a more thorough understanding of the pathogenesis of the disease.

Thus, these results seem to confirm the view held generally that children who suffer from this disease are predisposed by some unknown mechanism. Investigations of this aspect seem, however, to be sparse to judge from the literature.

Virologic Investigation (VIII)

Throat and faecal specimen from 87 non-diphtheritic croup cases were investigated for virus by the tissue culture technique. Tissue cultures of human embryonic lung were used. During this investigation, a virus was isolated, which seems to be different from other known viruses; this agent was named U-virus (III).

This agent was isolated in 30 per cent of the croup cases from either throat or faeces. Paired sera from the croup cases showed significant rises in haemagglutination-inhibiting antibodies between the two sera in 24 per cent, and in neutralizing antibodies in 22 per cent. Significant increases were recorded in 38 per cent in either of these two tests. Further, it was found that in the patients with this disease prednisone treatment seemed to entail an impaired antibody response against the U-virus.

In conclusion, the results indicated that an infection with U-virus had occurred simultaneously with the croup manifestations in about 40

per cent of the cases investigated. This does not, however, definitely imply that U-virus was the precipitating cause of the disease, but the results suggest a causal relation between U-virus and non-diphtheritic croup of subglottic type in a number of cases.

A group of children from day-nurseries and a control group of casualty cases from the Out-Patient Unit of the Surgical Department of the University Hospital with no recent history of respiratory disease of the latter group were selected for study simultaneously with the croup children. U-virus was isolated in 11.9 per cent in the day-nursery group, most of the strains being isolated in children with recent experience of mild respiratory disease. No virus was found in the casualty group. The serologic response against U-virus differed between the children in the day-nursery group and the croup children. Thus, in some cases the sera of the day-nursery children showed high titres of neutralizing antibodies but low titres of H-I antibodies. The croup children, however, generally showed high titres of H-I antibodies and low titres of neutralizing antibodies in their sera.

Evidence suggestive of a decrease in antibodies within 3 to 7 weeks in the cases infected with U-virus has been obtained (VI, VII). It was also found that the H-I antibody rise appears in advance of the neutralizing antibody rise and, further, that the H-I antibody titres may decrease prior to the neutralizing antibody titres (VII). The difference in antibody response in the day-nursery and croup-children may consequently be attributable to different days of sampling, but the fact that the majority of croup children had H-I antibodies already at the first sampling suggests other mechanisms to be involved.

Concluding Remarks Concerning Aetiology

Although the pathogenesis of non-diphtheritic croup of subglottic type is still obscure, evidence has been presented (21) suggesting bacteria to be of minor importance as the precipitating agent in most cases. Further, there is some virologic evidence of an association with acute laryngotracheobronchitis of influenza viruses (7) and CA-virus (2, 4).

An allergic factor in the croup syndrome has been proposed, although sparse evidence has been presented (11). A high percentage of non-diphtheritic croup has been observed in cases developing asthma of an infectious allergic type (8).

The results of the present investigation on the aetiology of non-diphtheritic croup appears to infer that:

1. Bacteria seem to be of minor importance as the precipitating agent in this disease although evidence of complicating bacterial infections has been adduced.
2. An association with an infection of a recently isolated virus, called

U-virus, has been shown to exist in about 40 per cent of the children attacked by this disease.
3. A non-infectious factor seems to be involved in the pathogenesis of this disease.

Thus the accumulated evidence seems to justify the assumption that non-diphtheritic croup of subglottic type may be attributed to common upper respiratory viruses. Further in the majority of cases a non-infectious factor, may be involved. This factor might be of an allergic nature but an evaluation of this factor must await a more thorough understanding of the pathogenesis of the disease.

Association of U-virus with Mild Respiratory Disease

Virologic study of an epidemic of upper respiratory disease in a day-nursery (VI)

In a day-nursery of Uppsala, 24 children under the age of 3 years contracted a respiratory disease during a two-week period at the end of August, 1957. The disease was characterized by cough, coryza, and fever (39 to 40°C) in most children (18 of 24) and by non-diphtheritic croup manifestations in 4 of them. Two of the children and 5 of the staff (including 9 persons) of the day-nursery as well as some family contacts reported upper respiratory infections with no fever during the period of the epidemic. Symptoms subsided in all cases within 3 to 4 days after the onset.

Pathogenic throat bacteria did not seem to have played any rôle as causative organisms. The specimens from throat and faeces yielded 9 virus strains on cultivation in tissue cultures of human embryonic lung in 8 of the 36 persons investigated. Of these strains, 2 were adenovirus types 1 and 5 respectively and the other 7 were found to be U-virus. All isolates were, however, obtained from faeces, except one from the throat of a child who fell ill 3 days prior to the sampling.

The analysis of the serologic response against U-virus in the children and the adults measured by H-I and neutralization tests indicated that in cases in which the onset preceded the date of initial sampling by 0 to 4 days the antibodies to the U-virus increased significantly between acute phase and convalescent serum samples. On the other hand, subjects who had shown symptoms 8–16 days before the initial specimens were taken showed in some cases a significant decline in antibody titres between the two sera taken at a fortnight interval.

Results of complement-fixation tests against influenza and adenovirus on the paired sera showed that these agents played no aetiologic rôle in this epidemic.

Thus, an infection with U-virus had occurred in a number of children

during the period of the epidemic. It was not possible to determine whether these infections were responsible for the respiratory symptoms observed, but it would appear that some correlation existed. This investigation together with the results of the transmission experiments with the U-virus in human adults (VII) suggest an association of this virus with mild respiratory disease according to the definitions of the Commission on Acute Respiratory Diseases (5). If this association was causal, the rapid decline in antibody titres between the two sera noted in 10 of 36 persons investigated seems remarkable, as for most other viruses isolated, no decline has been observed in neutralizing antibodies in a 3 to 5-week period after infection (3). The results of transmission experiments with "common cold" have, however, shown a period of immunity for 3 to 4 weeks after an infection (1). In this syndrome, it has not yet been established whether this period of immunity depends on circulating antibodies or on a refractive state of the tissue involved.

Transmission Experiments in Human Adults (VII)

This study was undertaken to find further evidence of an association of U-virus with mild respiratory disease. The subjects selected were patients in a mental hospital. The criteria for selection were good physical condition, no history of allergic manifestations, no experience of respiratory disease for 3 weeks prior to the start of the experiment, no antibodies against U-virus in either H-I or neutralization tests, and no deviation from "normality" in an oto-rhino-pharyngeal examination. Seven females were inoculated with U-virus and 5 females were chosen as controls, with the same standards for selection in these cases. The subjects were carefully investigated from clinical, virologic and bacteriologic aspects.

Clinical symptoms and/or signs developed within 1 to 3 days in 4 of the 7 infected subjects, permitting the diagnosis acute rhinitis and/or acute pharyngitis. No fever was recorded. In the controls, no signs or symptoms of respiratory tract infections occurred during the isolation period. In 2 of the controls, respiratory disease was observed after the isolation of the infected subjects were broken.

Bacteria did not seem to play any rôle in the aetiology of the symptoms and signs observed. The virologic investigation showed the U-virus to remain in the throat of the infected subjects for 3 to 9 days post inoculation and in faeces for 0 to 22 days post inoculation. The serum specimens showed a rise in both H-I and neutralizing antibodies within 2 weeks of inoculation and a significant decline after 7 weeks in all infected subjects. In the controls, none of the 5 patients showed antibodies against U-virus or U-virus at isolation attempts during the isolation period. Two of the controls contracted respiratory disease on the 11th post-inoculation day; 3 days after the isolation was broken. Virologic evidence was found that

one of these infections was due to U-virus. No significant rise in complement-fixing antibodies against influenza and adenovirus was recorded during this study in the sera of the subjects.

The results indicated that U-virus was the causative organism of the mild respiratory diseases observed in the infected subjects. The difficulty of establishing the diagnosis of mild respiratory disease even in investigations of this kind has, however, been stressed (5, VII). Numerous infectious and non-infectious conditions may cause the "common cold" syndrome, and they may be overlooked. The causal relationship between U-virus and mild respiratory disease must therefore be further evaluated.

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